Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 71 (2007) 82–86
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Note

Development of codominant simple sequence repeat, single nucleotide polymorphism and sequence characterized amplified region markers for the pea root rot pathogen, *Aphanomyces euteiches*

Hajime O. Akamatsu [a], Niklaus J. Grünwald [b], Martin I. Chilvers [a], Lyndon D. Porter [c], Tobin L. Peever [a,*]

[a] *Department of Plant Pathology, Washington State University, Pullman, WA 99164-6430, USA*
[b] *Horticultural Crops Research Laboratory, USDA-ARS and Department of Botany and Plant Pathology, Oregon State University, 3420 NW Orchard Ave., Corvallis, OR 97330, USA*
[c] *USDA-ARS, Washington State University Irrigated Agriculture Research and Education Center, 24106 N Bunn Rd., Prosser, WA 99350, USA*

Received 4 May 2007; received in revised form 1 August 2007; accepted 1 August 2007
Available online 9 August 2007

Abstract

Three kinds of genetic markers including simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) and sequence characterized amplified regions (SCARs) were developed from *Aphanomyces euteiches*. Of 69 loci tested, seven SSR, two SNP and two SCAR markers were codominantly polymorphic. These codominant markers and dominant markers described herein will facilitate population genetic and evolutionary studies of this important plant pathogen.

Keywords: *Aphanomyces euteiches*; Oomycete; *Pisum sativum* L.; Sequence characterized amplified region (SCAR); Simple sequence repeat (SSR); Single nucleotide polymorphism (SNP)

Aphanomyces euteiches Drechsler is an oomycete pathogen of leguminous crops and causes devastating root rot of pea (*Pisum sativum* L.) worldwide (Kraft and Pfleger, 2001). Amplified fragment length polymorphism (AFLP) analysis of isolates from the northwestern US revealed substantial genetic diversity within and between locations (Grünwald and Hoheisel, 2006). Past investigations of the population biology of *A. euteiches* relied on dominant markers such as random amplified polymorphic DNA or AFLP (Malvick et al., 1998; Grünwald and Hoheisel, 2006). Given that *A. euteiches* is a diploid organism, robust, codominant marker systems are needed for population genetic studies of this important plant pathogen.

Genomic DNA from isolates Ae01B13 and Ae02A9 (Table 1) were extracted from lyophilized mycelium using the Wizard Genomic DNA Kit (Promega). A plasmid library of *A. euteiches* genomic DNA was made using *Mbo*I-digested DNA and pBluescript SK-vector (Stratagene) digested with *Bam*HI, then dephosphorylated. In addition, a repeat sequence-enriched genomic library was constructed using the modified protocols of Moraga-Amador et al. (2001) and Kijas et al. (1994). DNA digested with *Mbo*I was ligated with *Sau*3AI linkers. PCR products amplified with a linker-specific primer were enriched for repeat sequences with streptavidin-coated magnetic particles (Promega) and biotin-labeled oligoprobes (Operon). Captured DNA fragments were cloned into the pDrive Cloning Vector in QIAGEN EZ Competent Cells (Qiagen). Recombinant bacterial clones were screened by hybridization with digoxigenin (DIG)-labeled repeat sequences [$(CA)_{15}$, $(CAA)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(AT)_{15}$, $(TAA)_{10}$, $(CG)_{15}$, $(GCC)_{10}$ and $(CAG)_{10}$] prepared using the DIG Oligonucleotide 3′-end Labeling Kit (Roche Applied Science). Alternatively, recombinant clones carrying 400-bp or larger inserts were screened by PCR with a combination of repeat sequence oligonucleotides, and SP6 or T7 primer (Operon). Plasmid DNAs were sequenced using SP6 or T7 primer

* Corresponding author. Tel.: +1 509 335 3754; fax: +1 509 335 9581.
E-mail address: tpeever@wsu.edu (T.L. Peever).

0167-7012/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.mimet.2007.08.001

Table 1
Aphanomyces isolates used in this study

Species	Isolate code[a]	Host	Location	Year[b]
A. euteiches	Ae01A5, **Ae01B13**, Ae01G5, Ae01G13, Ae01H2, Ae01H7, Ae01I2, Ae01J5	Pea (*Pisum sativum*)	Athena, OR	2001
	Ae02A4, **Ae02A9**, Ae02C2, Ae02D18, Ae02D20, Ae02F1, Ae02F8, Ae02G17	Pea (*P. sativum*)	Mount Vernon, WA	2002
	Roza-06-1	Pea (*P. sativum*)	Roza, WA	2006
	Spillman-06-1	Pea (*P. sativum*)	Pullman, WA	2006
	Mount Vernon-06-1	Pea (*P. sativum*)	Mount Vernon, WA	2006
	MN-06-1	Pea (*P. sativum*)	Stanton, MN	1994
A. cochlioides	Bern4C	Sugarbeet (*Beta vulgaris*)	MI	–
	Tx8	Sugarbeet (*B. vulgaris*)	TX	1997
	Tx51	Sugarbeet (*B. vulgaris*)	TX	1997

[a] Isolates Ae01B13 and Ae02A9 highlighted in bold text used for library construction.
[b] The year in which the isolates were collected. –: data not available.

and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on a PE Biosystems model 3700 automated DNA Sequencer (Applied Biosystems). Screening of non-enriched library revealed that no more than 0.2% of clones possessed repeat sequences. In contrast, 18% of colonies contained repeat sequences in the enriched library.

PCR primers for the markers were designed by eye to amplify genomic regions spanning the repeat motifs shown in Table 2. PCR consisted of 20 μl reaction volumes containing 20 ng template DNA, 1 × PCR buffer [20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100] (New England Biolabs), 0.2 mM each dNTP (New England Biolabs), 1 μM each forward and reverse primer and 1 unit of *Taq* polymerase (New England Biolabs). Thermal cycling was performed on a MyCycler thermocycler (Bio-Rad Laboratories) with 5 min denaturation at 95 °C followed by 40 cycles of 20 s at 95 °C, 20 s at an annealing temperature given in Table 2 and 30 s at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were electrophoresed on 3% high resolution sieving SFR agarose (Amresco) and visualized with ethidium bromide, separation of fragments differing in length by 3-bp was possible and confirmed by sequence analysis.

Sixty-nine primer pairs were screened for allelic variation against 20 US isolates of *A. euteiches* (Table 1). Fifty-seven of 69 primer sets generated a single PCR fragment from all isolates, except locus ApEu-2. Seven polymorphic codominant SSR markers ApEu-1, ApEu-2, ApEu-5, ApEu-7, ApEu-8, ApEu-9 and ApEu-10 could be scored on SFR agarose (Fig. 1 and Table 2). PCR primers for amplification of locus ApEu-2 generated two PCR fragments from 13 isolates, which suggested that these isolates were heterozygous at this locus. Six loci ApEu-3, ApEu-4, ApEu-6, ApEu-11, ApEu-24 and ApEu-25 (Tables 2 and 3) amplified PCR products from only some Washington State isolates, and may be used as dominant markers. The other monomorphic loci and minisatellite-like sequences found in the analyzed clones are deposited in the GenBank database (GenBank accession, EF122878 to EF122907). Sequence comparison of 15 loci among *A. euteiches* isolates revealed that 10 loci (ApEu-12 to ApEu-21) possess SNPs and two of them ApEu-12 and ApEu-13 were possible to be scored by PCR-restriction fragment length polymorphism (PCR-RFLP) with *Mse*I digestion and fragment separation on SFR agarose gel (Fig. 1 and Table 2). Locus ApEu-12 also contained an insertion/deletion (indel) of 2-bp, however the indel alone was not possible to score on SFR agarose. All of our screening isolates were homozygous for one or other form of the allele at locus ApEu-12. Locus ApEu-13 was scored against 20 isolates, 13 isolates including Ae01B13 produced a homozygous PCR-RFLP pattern (i.e. one allele type) of 376-, 34-, 3- and 297-bp, and seven isolates including Ae02A9 produced a heterozygous PCR-RFLP pattern (i.e. both allele types) of 376-, 342-, 34-, 31-, 6- and 297-bp, where the 376-bp fragment from one allele was cut by *Mse*I into 342- and 34-bp fragments. Loci ApEu-14 and ApEu-15 had alleles with 10- and 4-bp indels, respectively in certain isolates (Table 2), and amplification of the genomic region at ApEu-14 and ApEu-15 was scored on SFR agarose codominantly (Fig. 1). BLASTX searches (Altschul et al., 1990) showed significant similarity (*E*-value < e−14) of 11 clones including ApEu-10, ApEu-15 and ApEu-18 (Table 2) to genes from other organisms including enzymes and transporters (Table 3). Specificity of markers developed in this study was tested using three isolates of *Aphanomyces cochlioides* from sugarbeet (*Beta vulgaris* L.) (Table 1). PCR fragments were amplified from three loci ApEu-9, ApEu-10 and ApEu-14 out of 11 examined (Table 2), but no size differences were detected among the three isolates.

The loci cloned in the present study demonstrate low but detectable levels of polymorphism among Pacific Northwest USA isolates of *A. euteiches*. Screening the monomorphic loci identified in this study against other populations of *A. euteiches* will likely allow identification of additional polymorphic loci. These codominant and dominant markers will prove useful in characterizing genetic variation within and among *A. euteiches* populations.

Acknowledgement

The authors thank the USDA Cool Season Food Legume Research Program (Project 11W-3061-8301) for funding, Gwen-Alyn Hoheisel and L. Jacob Stone for technical assistance, Dr. David White for reviewing the manuscript, Dr. George Vandemark for *A. cochlioides* DNA, and Dr. Roxanne Mainz for *A. euteiches* isolates. The paper has been deposited as Plant Pathology New Series No. 0430, Department of Plant Pathology, College of Agricultural, Human, and Natural Resource Sciences; Agricultural Research Center, Project No.

Table 2
Codominant and dominant sequence characterized locus markers for genotyping *Aphanomyces euteiches* isolates

Type of marker	Locus	GenBank accession	Primer sequence (5'–3')	T_a [a]	Repeat motif of cloned allele [b]	Product size range	Cross reactivity [c]	*A*/*N* [d]	Additional information	Scorable on SFR agarose
SSR	ApEu-1	DQ915105	F: GCGACCAGAAGTGATATAGG R: AAGCGTGATTTGGAGCTCTC	53 °C	$(GT)_{13}(GA)_2$	175–185-bp	–	3/20		Yes
SSR	ApEu-2	DQ915106	F: TGATACGAATCATGCTCCGG R: ATACCTAGCCCCTTCAATGG	53 °C	$[(G/T)G]_6CA[G(T/G)]_5$	214-bp	–	2/20		Yes
SSR	ApEu-3	DQ915107	F: AATTTCGTCCTAGGACTCCC R: CGAGGTCAATTGGCAAATCC	53 °C	$(AT)_4$-$(CT)_6$	237-bp	nd	2/20	Dominant SSR	Yes
SSR	ApEu-4	DQ915108	F: CCAAATCGAACCAGCCAATG R: TCGCAAAGTACCATCCAAGG	53 °C	$[(A/C)(A/C)]_{18}$	244-bp	nd	2/20	Dominant SSR	Yes
SSR	ApEu-5	DQ915109	F: TCACCGCCATAGCATTACTC R: ATCGAACGAATGACCAACGG	50 °C	$(TTG)_3$-$(TTG)_2$-$(TTG)_3$-$(TTG)_2$-$(TTG)_3$-$(TTG)_4$	206–209-bp	–	2/20		Yes
SSR	ApEu-6	DQ915110	F: TTTCGTCGCTGGGTCCTTCT R: AAGGACTTCTGGACTTCTGC	53 °C	$(CAA)_6$	159-bp	nd	2/20	Dominant SSR	Yes
SSR	ApEu-7	DQ915111	F: TTACTCACATTCCCCTGTC R: TGCGAAGCAAGAAAGGTTG	50 °C	$[(C/T)TG]_8[(C/G)AG]_2[(C/T)T(G/C)]_8$	214–217-bp	–	2/20		Yes
SSR	ApEu-8	DQ915112	F: GTACTTCGAACTTCGTGAG R: CTTCTGTCCAAACAGAGTGC	60 °C	$[CAG(C/T)(A/T)ACAGCA(C/A)C(A/G)A]_9$	288–378-bp	–	2/20		Yes
SSR	ApEu-9	DQ915113	F: CAAGGAAGAGAAGCCTCAAG R: CAGTTGATGAAGACGCTGAC	53 °C	$[CA(G/A)]_7CTT[CA(G/A)]_8CTT[CA(G/A)]_9(CTG)_2[(C/A)AA]_9[CA(A/G)]_{13}$	199–220-bp	+	2/20		Yes
SSR	ApEu-10	DQ915114	F: ACGTGACGGAGATGGATGTC R: CAAACGCAGGCTTCTTTTGC	53 °C	$[C(G/A)(G/A)]_4C(CAA)_3C(CAA)_4$-$(GTT)_3(CAA)_3$	195–204-bp	+	2/20		Yes
SSR	ApEu-11	DQ915115	F: TGCGTACGCCCGCATTTCTG R: CCGATACTAGTAGTTGTTAACC	53 °C	$[GTTGGTG(C/T)TGG(C/T)]_5$	191-bp	nd	2/20	Dominant SSR	Yes
SNP-RFLP	ApEu-12	DQ915116	F: GGTCACCTGACTCACACAAA R: GGAGCTCACTTTCATACTCC	60 °C	$(CA)_3AAA(CA)_3CGGA(CA)_3$-$(C)_7$-$(C)_7$	474–476-bp	–	2/20	SNPs [e, f, g] and indels 38 (C/T), 44 (G/C), 139 (A/G), 216 (A/T), 292 (A/G)	Yes
SNP-RFLP	ApEu-13	DQ915117	F: AACATGCGACATCAAGGCAC R: AGCCATTGCGACGAGTTTCT	60 °C	$(ACAAA)_2CAT(AC)_2CAA(TCGTCTCA)_2(TA)_2(CA)_2$	709-bp	–	2/20	SNPs[e,f,g] 121 (A/C), 124 (C/A), 125 (A/G), 140 (T/A), 217 (A/C), 236 (C/T), 277 (T/C), 315 (G/A), 338 (T/A), 353 (C/T), 525 (A/G)	Yes

SCAR	ApEu-14	DQ915118	F: CTCGTCATCACCATGGTGAA R: GTGTCCACAAGGAAGAATCG	60 °C	$(CA)_2(GC)_2$ G$(TGC)_2$AC $(AGA)_3C(AG)_3$	228–238-bp	+	2/20	SNPs[f,g] and indels 177 (G/A), 210 (A/G)	Yes
SCAR	ApEu-15	DQ915119	F: TCCTTCATCAAGTCGTTGG R: AATGCCAACTTGAACGAGC	50 °C	$(GAG)_2(GTT)_2$-$[(G/T)(G/T)T]_6$-$(AGT)_2$ $[G(T/A)T]_3$	211–215-bp	–	2/20	SNPs[f,g] and indels 258 (G/A), 265 (T/G), 309 (T/A)	Yes
SNP	ApEu-16	EF122863	F: AGCATGTTGGTAGGACAAGC R: ACCGCCTAGACTATTAGGGA	60 °C	$(GT)_3$-$[G(T/A)]_3(GTG)_2$-$(GT)_3(AGTG)_2$-$[G(T/A)]_3(ATGGT)_2$	233-bp	nd	2/6	SNPs[f, h] 203 (G/C), 254 (G/A), 255 (A/T)	No
SNP	ApEu-17	EF122864	F: AAGCGTTTCGACCCACTTGA R: AATCCTTAACACGCCAGTGC	60 °C	$[C(T/A/G)T]_{13}$	234-bp	nd	2/6	SNPs[f,h] 361 (C/T), 394 (G/A)	No
SNP	ApEu-18	EF122865	F: CTTTTGCATAAAGAGCGCCG R: TCCAGAGGAACTACGTCAAC	60 °C	$[(G/A)A]_9$-$(G)_7$-$(G)_6$-$(G)_9$	559-bp	nd	2/4	SNPs[f,g] 252 (A/G), 295 (T/G)	No
SNP	ApEu-19	EF122866	F: CTTTACTTTGAACACGGCCA R: CGACTTTCAGCATATCATCG	60 °C	$(GAC)_2TT(ACGAAA)_2(CAG)_3$ $[TGCC(A/G)]_2$	213-bp	nd	2/6	SNPs[f,h] 146 (C/T), 170 (T/C), 178 (C/T)	No
SNP	ApEu-20	EF122867	F: ATCACCACATCGTCGACAGT R: AAGTGCAGTCGCTTCAAGTG	60 °C	$(GCTT)_2CGG[C(C/A)T]_4$	182-bp	nd	2/4	SNPs[f,g] 117 (T/C), 141 (C/T)	No
SNP	ApEu-21	EF122868	F: CCAAGAGTCATGCAAATGCC R: ACCATGAGAAACTTCGACCC	60 °C	$(GA)_2(A)_7$-$(G)_5$	226-bp	nd	2/6	SNPs[f,h] and indels 163 (A/C)	No

[a] Annealing temperature for each primer set.
[b] Intervening nucleotide sequence indicated by (–).
[c] Cross reactivity of primers with DNA from *A. cochlioides*: +, PCR product-amplified;–, not amplified; nd, not determined.
[d] Number of alleles/Number of individuals genotyped.
[e] SNP loci scored by PCR-RFLP with *Mse*I.
[f] SNP position (identity) is indicated. Frequency of each SNP allele was 0.5.
[g] SNP identity determined using isolates Ae01A5 and Ae01B13 from Oregon State and isolates Ae02A4 and Ae02A9 from Washington State.
[h] SNP identity determined using isolates Ae01A5, Ae01B13, Ae01G5 from Oregon State and isolates Ae02A4, Ae02A9, Ae02G17 from Washington State.

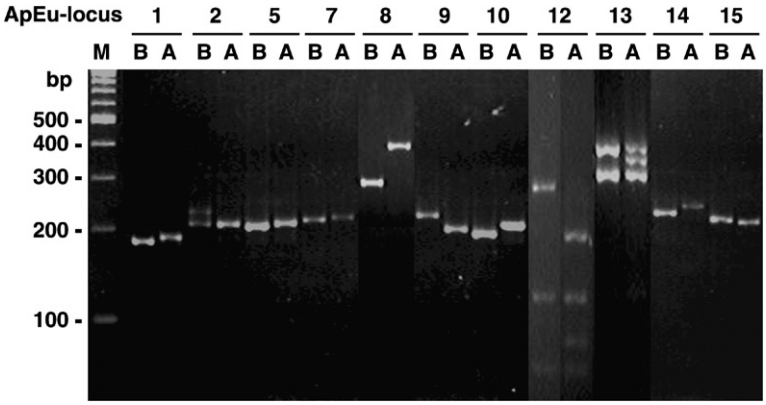

Fig. 1. Eleven codominant polymorphic markers developed from *Aphanomyces euteiches* and scored by electrophoresis on super fine resolution (SFR) agarose gel. The number of ApEu-locus corresponds to that in Table 2. ApEu-loci 1, 2, 5, 7, 8, 9 and 10 are simple sequence repeat (SSR) markers, ApEu-loci 12 and 13 are single nucleotide polymorphism (SNP) markers assessed by restriction fragment length polymorphism (RFLP) and ApEu-loci 14 and 15 are sequence characterized amplified region (SCAR) markers. Lane M is 100-bp ladder (New England Biolabs), lanes labeled with B are isolate Ae01B13, and lanes labeled with A are isolate Ae02A9.

Table 3
Aphanomyces euteiches clones with significant similarity to protein sequences in non-redundant database

Locus	GenBank accession	Sequence length	BLASTX match [a] Similarity to	Organism	BLASTX *E*-value [b]
ApEu-10	DQ915114	602-bp	Endo-1,4-β-xylanase	*Clostridium thermocellum*	2e−24
ApEu-15	DQ915119	342-bp	NADH flavin oxidoreductase/NADH oxidase	*Vibrio alginolyticus*	9e−20
ApEu-18	EF122865	591-bp	Amino acid permease-associated protein	*Flavobacterium johnsoniae*	6e−20
ApEu-22	EF122869	192-bp	Polygalacturonase	*Phytophthora cinnamomi*	2e−21
ApEu-23	EF122870	425-bp	Exocellulase	*Irpex lacteus*	5e−38
ApEu-24 [c]	EF122871	568-bp	Subtilisin-like serine proteinase	*Aphanomyces astaci*	2e−22
ApEu-25 [d]	EF122872	436-bp	Trypsin proteinase	*Aphanomyces. astaci*	4e−29
ApEu-26	EF122873	213-bp	Cell wall-associated hydrolase	*Actinobacillus succinogenes*	7e−23
ApEu-28	EF122875	560-bp	Metalloprotease	*Aedes aegypti*	4e−14
ApEu-29	EF122876	415-bp	Pleiotropic drug resistance transporter	*Phytophthora sojae*	6e−15
ApEu-30	EF122877	504-bp	Fatty acid synthase α-subunit	*Lachancea kluyveri*	2e−43

[a] Sequences producing the most significant alignment with those of *A. euteiches* and the corresponding organisms in the database.
[b] *E*-value calculated by BLASTX analysis at NCBI.
[c] PCR primers (5'-GATCAGACGTCGTGGATTTG-3′ and 5′-TACAACACCATCTCGGGGAC-3′) amplify 429-bp product only from some Washington State isolates.
[d] PCR primers (5′-TCTATCACGACCATCCAACC-3′ and 5′-TGACCCTTCAAACCACCATC-3′) amplify 210-bp product only from some Washington State isolates.

WNP00300, Washington State University, Pullman, WA 99164-6430, U.S.A.

References

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Grünwald, N.J., Hoheisel, G.-A., 2006. Hierarchical analysis of diversity, selfing and genetic differentiation in populations of the oomycete *Aphanomyces euteiches*. Phytopathology 96, 1134–1141.

Kijas, J.M.H., Fowler, J.C.S., Garbett, C.A., Thomas, M.R., 1994. Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16, 657–662.

Kraft, J.M., Pfleger, F.L., 2001. Compendium of Pea Diseases and Pests, 2nd ed. The American Phytopathological Society Press, St. Paul, Minnesota, USA.

Malvick, D.K., Grau, C.R., Percich, J.A., 1998. Characterization of *Aphanomyces euteiches* strains based on pathogenicity tests and random amplified polymorphic DNA analyses. Mycol. Res. 102, 465–475.

Moraga-Amador, D., Farmerie, B., Brazeau, D., Clark, G., 2001. Tools for Developing Molecular Markers. Interdisciplinary Center for Biotechnology Research. The University of Florida, Gainesville, Florida, USA.